

The Inhalation Toxicology of Benzene: Incidence of Hematopoietic Neoplasms and Hematotoxicity in AKR/J and C57BL/6J Mice

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The Inhalation Toxicology of Benzene: Incidence of Hematopoietic Neoplasms and Hematotoxicity in AKR/J and C57BL/6J Mice. SNYDER, C. A., GOLDSTEIN, B. D., SEL-LAKUMAR, A. R., BROMBERG, I., LASKIN, S., AND ALBERT, R. E. (1980). *Toxicol. Appl. Pharmacol.* **54**, 323-331. AKR/J mice and C57BL/6J mice were given lifetime exposures to 100 and 300 ppm benzene, respectively. Peripheral blood cell counts were obtained bi-weekly throughout the exposures. Anemia and lymphocytopenia were produced in benzene-exposed AKR mice. Twenty percent of the exposed AKR mice developed bone marrow hypoplasia, compared to 2% for the controls. The benzene exposures did not alter the incidence or induction time of the viral-induced lymphomas commonly seen in AKR mice. In C57BL mice, exposure to benzene produced anemia, lymphocytopenia, and neutrophilia accompanied by a left shift. Thirteen (33%) of the exposed C57BL mice developed bone marrow hyperplasia and in four of these mice, hyperplasia was essentially limited to granulopoietic elements. None of the control C57BL mice developed bone marrow hyperplasia. In benzene-exposed C57BL mice there was a significant increase in the incidence of hematopoietic neoplasms including six cases (15%) of thymic lymphoma. Although two control mice (5%) died with lymphoma neither of these tumors involved the thymus. Thymic lymphoma is rare in C57BL mice but can be produced by ionizing radiation and chemical carcinogens.

Human exposure to benzene has been associated with pancytopenia and its variants (Aksoy *et al.*, 1971, 1972, 1976; Mallory *et al.*, 1939; Pollini *et al.*, 1969; Saita and Sbertoli, 1954) and acute myelogenous leukemia and its variants (Aksoy *et al.*, 1976; Saita and Vigliani, 1962; Tareeff *et al.*, 1963; Vigliani and Saita, 1964). The cytopenic effects of benzene exposure have been readily reproduced in animals, most often by Parenteral injection (Gerarde and Ahlstrom, 1966; Latta and Davis, 1941; Minai, 1967; Selling, 1916; Weiskotten *et al.*, 1916). Except for work performed in this labora-

tory (Snyder *et al.*, 1978a) there appear to be no reports of the experimental production of benzene-induced myelogenous leukemia. Inhalation studies have, for the most part, been of short duration and/or have involved acute or subacute exposure levels (Boje *et al.*, 1970; Deichmann *et al.*, 1963; Jenkins *et al.*, 1970; Nau *et al.*, 1966; Uyeki *et al.*, 1977). In addition, a great deal of previous work has produced confusing and often contradictory results regarding both the cytopenic and proliferative effects of benzene exposure (Laskin and Goldstein, 1977).

The purpose of our studies is to acquire hematological dose-response information

¹Deceased.

from lifetime, moderate-level, inhalation exposures of rodents. Attempts have been made to choose exposure levels that would produce significant hematological damage but would not 'greatly' shorten survival. In this way, the peripheral blood cell responses of a number of different species and/or strains exposed to benzene vapor could be characterized perhaps leading to a clearer picture of experimental benzene-induced hematotoxicity.

The first series of studies involved AKR mice and Sprague Dawley rats exposed to 300 ppm benzene (Snyder *et al.*, 1978b). This exposure level caused high mortality, lymphocytopenia, anemia, neutrophilia, and reticulocytosis in the mice; but only lymphocytopenia in the rats. We now wish to report on AKR mice exposed to 100 ppm and C57BL mice exposed to 300 ppm. The severity of the peripheral blood cell responses generated by exposures of AKR mice to 300 ppm prompted the lifetime study of these mice at 100 ppm. The C57BL strain was chosen to determine if AKR mice were unique or if another mouse strain would also be more susceptible than rats to the hematotoxic effects of benzene. A further purpose was to study the effect of chronic benzene exposure on the lymphoma incidence in these strains. The AKR strain carries a virus that spontaneously produces a high incidence of lymphoma and kills most of the affected mice within 1 year (Kahn and Novak, 1973). The C57BL strain carries a virus that has been shown to yield a high incidence of lymphoma following exposure to radiation, carcinogens, and immunosuppressive agents (Kaplan, 1967; Igel *et al.*, 1969; Imamura *et al.*, 1973).

METHODS

Exposure

Male, 6-week old, AKR/J and C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were quarantined and observed for anomalous behavior

for 2 weeks. Animals were then randomly distributed into test and control groups. There were 50 mice in each of the AKR test and control groups and 40 mice in each of the C57BL test and control groups. Exposures were conducted in stainless-steel, dynamic exposure chambers (Drew and Laskin, 1973). Test animals were exposed for 6 hr/day $\times$ 5 days/week for life to either 100 ppm benzene (AKR) or 300 ppm benzene (C57BL). Sham control animals were exposed to filtered conditioned air in a duplicate chamber during the same time periods as exposure animals. Chamber exposures continued until the last test animal died. Any surviving control animals were then allowed to live out their lives without further air-sham exposures. Methods of animal housing and handling as well as exposure methods including generation and analysis of test atmospheres were as previously described (Snyder *et al.*, 1978b). Briefly, benzene atmospheres were generated by feeding air with entrained benzene vapor into the exposure chamber. Benzene concentrations in chamber air were determined every 30 min during the daily exposures using an ultraviolet spectrophotometric technique.

Animals were clinically observed daily for evidence of morbidity. Weight determinations were performed weekly for the first 4 weeks and biweekly thereafter.

Blood Parameters

Venous tail blood from 10 test and 10 control mice of each strain was used for all determinations. Test and control animals were bled every other week on the same days within a period of 1.5 hr to minimize differences caused by handling, circadian rhythms, etc. Only males were used in the study in order to avoid anomalous white counts due to estrus. In the C57BL study, as mortality increased, mice from each group (either benzene exposure or air exposure) were cycled into the respective groups of mice undergoing the bi-weekly blood cell monitoring so that throughout most of the study there were always 10 mice in each of the test and control groups used for blood cell counts. Red cell, white cell, and white cell differential counts were performed as previously described (Snyder *et al.*, 1978b). Individual absolute neutrophil and lymphocyte counts were determined by multiplying the percentage of mature cells of each type in the differential count of a given animal by the total white cell count for that animal.

Pathology

Tissues routinely sectioned from animals dying during the study included lung, liver, spleen, kidney, and bone marrow, as well as all abnormally appearing

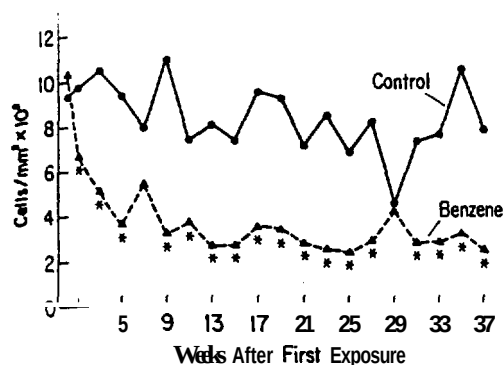


FIG. 1. Lymphocyte counts of AKR mice. An asterisk denotes lower by statistically significant differences (± 2 SE).

organs. These sections were fixed in formalin, embedded in paraffin, sectioned at $5\ \mu\text{m}$ mounted on slides and stained with hematoxylin and eosin. All slides were reviewed by the same pathologist.

RESULTS

AKR Mice at 100 ppm

The chronic exposure of these mice ended after 505 days with the death of the last test animal. There were a total of 337 exposures at an integrated mean benzene concentration of 100 ppm. There were no statistically significant differences between test and control mice in median survival (39 and 41 weeks, respectively) or in rate of weight gain. Hematological monitorings were suspended after 37 weeks because too few animals remained due to advancing mortality.

Statistically significant² lymphocytopenia was observed in treated mice vs controls after 1 exposure week and this trend continued throughout the exposure (Fig. 1). Lymphocyte depressions in exposed mice, however, were not as severe as those observed in AKR mice exposed to 300 ppm (Nyder et al., 1978b). After the start of exposure red cell levels of treated animals were consistently depressed relative to

² ± 2 standard errors (± 2 SE) is the criterion used for statistical significance in the differences between mean blood counts of test and control animals.

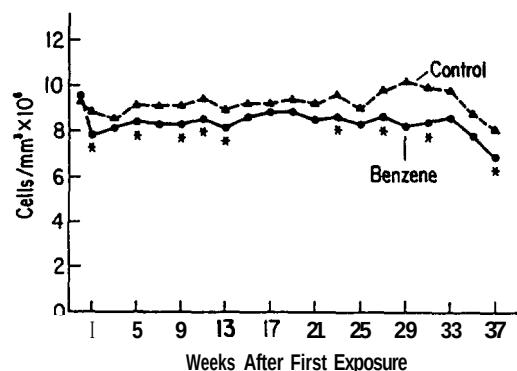


FIG. 2. Red blood cells of AKR mice. An asterisk denotes lower by significant differences (± 2 SE).

controls and these depressions were statistically significant at 9/19 monitoring periods (Fig. 2). Test mice showed some tendency toward neutrophilia throughout the exposure, however, neutrophil levels were elevated by statistically significant amounts in only 3/19 monitoring periods (Fig. 3). Little or no morphological differences were observed in the peripheral blood of test and control animals.

Malignant lymphoma was confirmed in 29/49 treated animals³ and in 24/50 controls. Disseminated, lymphocytic lymphoma with thymic involvement was the predominant type of lymphoma found in both exposed and control mice. Of the 29 exposed mice found with lymphoma, 15 exhibited lymphoma cell infiltrates in the bone marrow,

³ One animal was removed from the at risk pool after it was found outside of its living quarters.

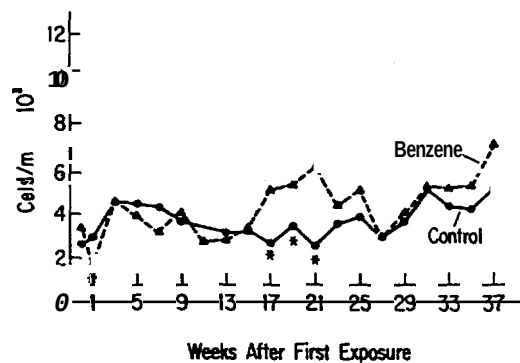


FIG. 3. Neutrophil counts of AKR mice. An asterisk denotes lower by statistically significant differences (± 2 SE).

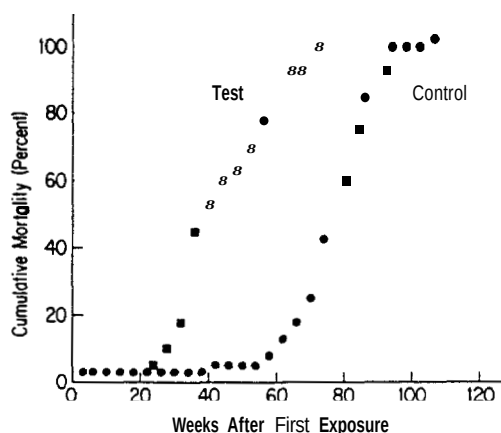


FIG. 4. Comparison of cumulative mortality between C57BL mice exposed to 300 ppm benzene and air controls. Only every other datum point is shown.

7 were found with hypoplastic bone marrows, and 7 were found with essentially normocellular bone marrows. Of the 24 control mice found with lymphoma, 5 exhibited lymphoma cell infiltrates in the bone marrow; 1 was found with bone marrow hypoplasia, and 18 mice were found with essentially normocellular bone marrows.

Data on lymphoma occurrence was analyzed by the log rank method (Peto, 1974; Peto and Pike, 1973; Peto *et al.*, 1977) which allows for comparison of tumor incidence and tumor induction time between two or more groups corrected for survival. No statistical difference in lymphoma type or occurrence was found between exposed and control AKR mice.

Bone marrow hypoplasia was found in a total of 10 treated mice (20%) but in only one control. Bone marrow hypoplasia in these mice was manifested by a diminution of all formed elements, and the appearance of a few clasmotocytes and areas of hemorrhage. The difference in the incidence of bone marrow hypoplasia found in exposed and control mice was significant at the $p < 0.05$ level ($\chi^2 = 4.81$). The 20% incidence found in animals exposed to 100 ppm is substantially less than the 81% incidence of bone marrow hypoplasia found in AKR mice exposed to 300 ppm (Snyder *et al.*, 1978b).

Most of the animals in both exposed and control groups developed respiratory infections (bronchitis, pneumonia) and some showed liver necrosis but it is difficult to determine whether these conditions were the cause of death in each case.

C57BL Mice at 300 ppm

The chronic exposure of C57BL mice was completed after 488 days with the death of the last test animal. There were a total of 331 exposures at an integrated mean concentration of 300 ppm. After the start of exposures treated mice had a median survival of 41 vs 75 weeks for controls (Fig. 4) and showed weight gain depression relative to controls throughout the study (Fig. 5). Acquisition of peripheral blood data was suspended after 61 weeks because too few animals were alive in the test group.

After the start of exposures, test mice exhibited a statistically significant degree of lymphocytopenia in 29/30 monitoring periods and a statistically significant degree of anemia relative to controls in 30/30 monitoring periods. These differences began after 1 exposure week and continued throughout the course of the study (Figs. 6 and 7). Neutrophilia was evident in test mice vis-a-vis controls after 17 weeks and the degree of neutrophilia was statistically

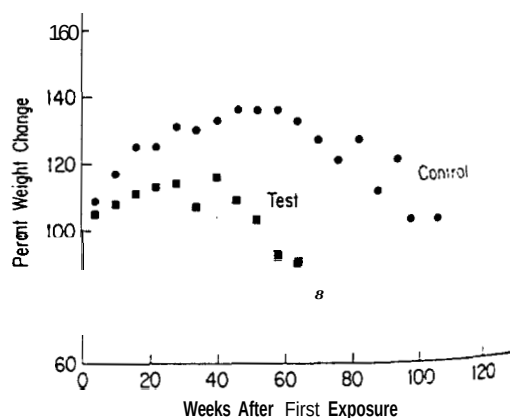


FIG. 5. Comparison of percentage weight change between C57BL mice exposed to 300 ppm benzene and air controls. Only every other datum point is shown.

significant in 15 of the 22 remaining monitoring periods (Fig. 6).

As exposures progressed, qualitative changes were observed in the peripheral blood cell morphology of test animals. Anisocytosis appeared after 4 exposure weeks and poikilocytosis began to appear after 15 weeks. Hyperlobulated, mature neutrophils were observed and a neutrophilic left shift appeared concurrently with the neutrophilia. The left shift was signaled, at first, by the appearance of elevated levels of metamyelocytes and progressed in intensity until, by the end of the first year, myelocytes and promyelocytes began to appear. Giant platelets also appeared by the end of the first exposure year. Similar changes were not observed in the peripheral blood of control animals.

Histological evaluations revealed an increased incidence of hematopoietic neoplasms as well as bone marrow and splenic hyperplasia in exposed mice. These findings are presented in Table 1. The eight exposed animals dying with hematopoietic neoplasms were alive for an average of 262 days after the start of exposure (first, 213 days; last, 344 days). Six of the exposed mice died, with lymphocytic lymphoma, one died with plasmacytoma (myeloma), and

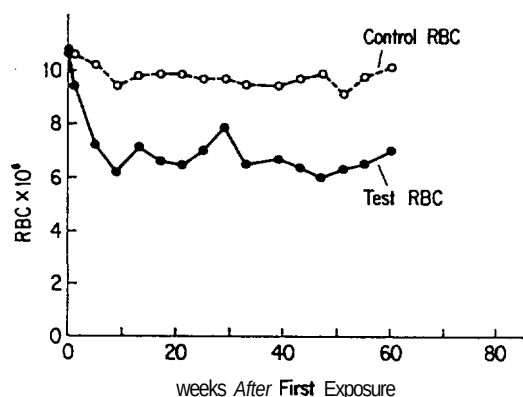
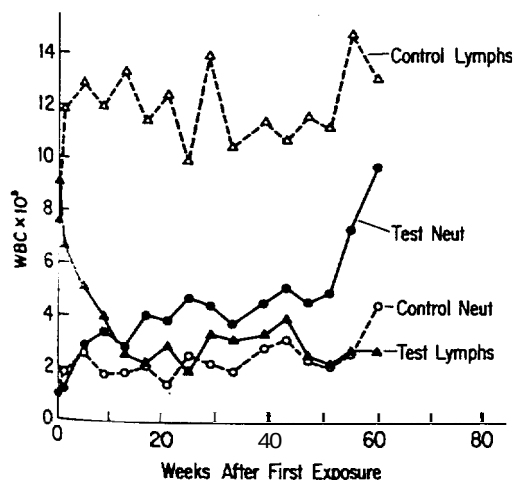


FIG. 7. RBC of C57BL mice. Only every other datum point is shown. All points after start of exposure are different by statistically significant amounts (± 2 SE).

one died with a leukemia in which the predominant cell type appears to be a hematocytoblast. Two control animals died after 282 and 608 days with lymphocytic lymphoma. There was thymic involvement in all six of the lymphocytic lymphomas found in benzene-treated mice. In contrast, there was no thymic involvement in the two control mice dying with lymphoma. An actuarial plot of the incidence of hematopoietic neoplasms in both groups is given in Fig. 8. The increase in the overall incidence of hematopoietic neoplasms in exposed mice is statistically significant by the log rank method at the $p < 0.005$ level, $\chi^2 = 9.81$. When lymphoma incidence is compared in both groups, the increase in exposed mice is significant at the $p < 0.01$ levels, $\chi^2 = 7.06$. If only the incidence of thymic lymphoma is compared in both groups, the increase in exposed mice is significant at the $p < 0.005$ level, $\chi^2 = 9.70$.

Comparison of those animals in both groups dying without evidence of neoplasia shows highly significant increases ($p < 0.001$) of bone marrow hyperplasia ($\chi^2 = 16.37$) and spleen hyperplasia ($\chi^2 = 15.93$) in exposed mice. Of the thirteen cases of bone marrow hyperplasia in exposed mice, four involved proliferation of granulocytic cells while the remainder consisted of a general hyperplasia which, although predominantly myeloid in nature, involved all



6. WBC of C57BL mice. Neut, mature neutrophils; lymphs, mature lymphocytes. Only every-other datum point is shown.

TABLE 1
HISTOLOGICAL EVALUATIONS OF C57BL MICE
EXPOSED TO 300 ppm BENZENE AND OF AIR SHAM
CONTROLS

	Incidence	
	Test	Control
1. Hematopoietic neoplasms	8/40	2/40
2. Bone marrow hyperplasia without evidence of hemato- poietic neoplasm	13/32	0/38
3. Spleen hyperplasia without hematopoietic neoplasm	16/32	2/38

formed elements. The splenic hyperplasia was due, predominantly, to ectopic hematopoiesis. Respiratory infections, especially pneumonia, were found in nearly all exposed and control mice, however, it would be difficult to pinpoint the exact cause of death in each case.

DISCUSSION

The peripheral blood cell effects observed in this study are qualitatively similar to those reported in the previous study (Snyder *et al.*, 1978b). At 300 ppm, some cytopenic effects have been produced in all animals (Sprague-Dawley rats, AKR mice and C57BL mice). At this concentration, all animals exhibited peripheral lymphocytopenia and the mice also exhibited anemia. At 300 ppm, the AKR mice were more sensitive to the peripheral cytopenic effects of the exposure to benzene than the C57BL strain. Sprague-Dawley rats, on the other hand, were less sensitive to the effects of benzene than either mouse strain, exhibiting milder lymphocytopenia and only a trend to anemia. The AKR mice gave evidence of a dose-response effect since the degree of lymphocytopenia and anemia at 100 ppm were not as severe as those observed at 300 ppm. At 300 ppm, the red cell levels of these animals declined to $6 \times 10^6/\text{mm}^3$ whereas at 100 ppm, the levels

were about $8 \times 10^6/\text{mm}^3$. Likewise, lymphocyte counts declined to less than 1000 mm^3 at 300 ppm but only to about 3000 mm^3 at 100 ppm. Nevertheless, the cytopenic effects observed at 100 ppm do represent statistically significant differences from control peripheral blood cell values.

Neutrophilia has been produced in both AKR and C57BL mice following exposure to 300 ppm benzene. A dose-response effect is evident in the AKR strain since the neutrophilia was not as marked at 100 ppm as at 300 ppm (Snyder *et al.*, 1978b). In view of the cytopenic effects of the exposures on peripheral lymphoid and erythroid cells, the proliferative effects on the granulocytic cell line represent a sharp contrast in response. To our knowledge, there has been only one report of benzene-induced myelogenous leukemia in animals (Snyder *et al.*, 1978a) even though there is a direct association between benzene exposure and myeloid leukemia in humans (Laskin and Goldstein, 1977). The production of these granuloproliferative responses in these exposed mice may be meaningful, especially in view of the concurrent left shift and myeloid hyperplasia observed in exposed C57BL mice.

There is a marked contrast in the effects of benzene exposure on the occurrence of lymphoma in the AKR and C57BL strains. At 300 ppm, the survival of the AKR mice

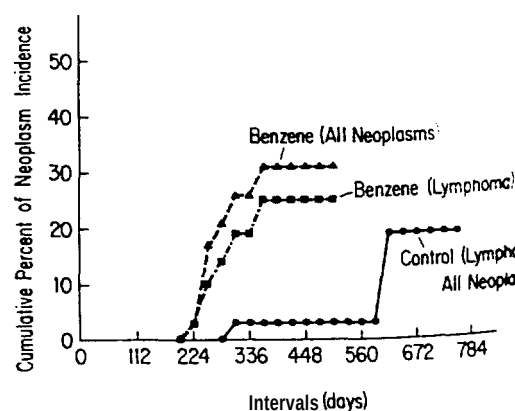


FIG. 8. Actuarial plot of hematological neoplasm incidence in C57BL mice.

adequate to allow an evaluation of the effects of benzene on lymphoma induction. Median survival of 300-ppm-treated animals was only 3 months (Snyder *et al.*, 1978b). In the 100-ppm study, median survival of treated AKR mice was 39 weeks which is almost identical to our control and reported control values (Kahn and Novak, 1973). No effects on the incidence or type of lymphoma or on induction time was evident; however, in AKR mice exposed to 100 ppm benzene.

On the other hand, there was a significant increase in hematopoietic neoplasms, particularly thymic lymphomas, in C57BL mice exposed to 300 ppm benzene. This increase occurred despite the shorter survival of exposed mice compared to controls.

Thymic involvement was evident in all six cases of lymphocytic lymphoma found in exposed mice but was not evident in either case of lymphocytic lymphoma in control mice. Thymic lymphoma usually develops in C57BL mice in association with a virus following exposure to ionizing radiation, chemical carcinogens (Kaplan, 1967) or the immunosuppressant azathioprine (Imamura *et al.*, 1973). In contrast to AKR mice, spontaneous thymic lymphoma is apparently rare in C57BL mice (Dunn and Deringer, 1968).

The depression in circulating lymphocyte levels observed in these studies may indicate that benzene is an immunosuppressant. The neoplasm incidence and induction time produced in C57BL mice by chronic exposure to 300 ppm benzene closely resembles those produced in these animals following chronic administration of azathioprine (Imamura *et al.*, 1973). This may strengthen the argument that a reduction in immune competence may be a requirement for the production of lymphomas in these animals.

C57BL mice have been the subject of a study by Ward *et al.* (1975) in which benzene was administered subcutaneously over a 54-week period. The hematopoietic neoplasm incidences of control groups in this study differ from the findings of our study.

In the Ward *et al.* study, hematopoietic tumors appeared in both the benzene-injected and control animals in the second year after the start of the experiment and incidences were higher in the control groups (25%, 5/20) than in the test group (17.5%, 14/80). In addition, Ward *et al.* reported no incidences of thymic lymphoma in either the benzene-treated or control groups. Thymic lymphoma was produced in a positive control group receiving nitrosobutylurea.

The overall hematopoietic tumor incidence in benzene-treated mice in the Ward *et al.* study is in good agreement with the incidence observed in the benzene-exposed mice in our study. There is a contrast, however, between the low neoplasm incidence (5%) in the control mice of our study and the relatively high incidence (25%) in the controls of the Ward study. The reported spontaneous lymphoma incidence for C57BL mice appears to be quite low. Igel *et al.* (1969) found a 6% (4/62) spontaneous incidence at 20 mos; Lieberman and Kaplan (1959) reported a 1.4% (1/74) incidence at 600 d; and Myers *et al.* (1970) found a 0% (0/370) incidence at 7 months. Since the benzene-exposed, tumor-bearing mice in our study all died within a time frame when these various authors (including Ward *et al.*) report a very low or zero spontaneous incidence, it appears that the benzene inhalation has increased the incidence of hematopoietic neoplasms and shortened induction time.

The difference in route of administration may explain why no thymic lymphomas were produced by Ward *et al.* in contrast to our own study. Injury to the bone marrow and spleen as well as the thymus is apparently required to produce thymic lymphoma in these animals (Kaplan, 1967) and a systemic distribution of benzene would seem to be necessary to produce injuries to all of these organs. Subcutaneous injection may not produce the systemic distribu-

tion of benzene that inhalation exposure would be expected to produce.

In summary, the results of our two studies indicate that, for a given exposure level, the two strains of mice tested are more susceptible to the cytopenic effects of inhaled benzene than Sprague-Dawley rats, and AKR mice are more sensitive than C57BL mice. These mice also respond to the exposures with a neutrophilia, and, in the C57BL strain, this is accompanied by a strong left shift and bone marrow hyperplasia. Thus, a proliferative effect of benzene exposure on the myeloid cell line has been produced and this is the cell line that undergoes leukemic transformation in most cases of human leukemia associated with benzene exposure (Laskin and Goldstein, 1977). The exposures seem to have little or no effect on the spontaneous lymphoma of the AKR strain but there appears to be an effect, at 300 ppm, on the inducible lymphoma of the C57BL strain leading to an increased incidence of hematopoietic tumors with decreased induction times. The production of thymic lymphoma in C57BL mice indicates that inhaled benzene acts on these animals in a manner similar to other tumorigenic agents.

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